Original Article

GJB2 mutation spectrum in Inner Mongolia and its comparison with other Asian populations

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Abstract Mutations in the GJB2 gene are the most frequently found mutations in patients with nonsyndromic hearing impairment. However, the mutation spectrum and prevalence of mutations vary among different ethnic groups. Every year, 30, 000 babies are born with congenital hearing impairment in China. In order to provide appropriate genetic testing and counseling to the family, we investigated the molecular etiology of nonsyndromic deafness in 135 unrelated school children attending Chifeng Municipal Special Education School in Inner Mongolia, China. The coding exon of the GJB2 gene was PCR amplified and sequenced. In addition, the 12S rRNA gene and $tRNA^{ser(UCN)}$ of mitochondrial genome were screened for mutations responsible for hearing impairment. Sixty four GJB2 mutant alleles, including 60 confirmed pathogenic alleles and 4 unclassified variants, were identified in 31.1% (42/135) of the subjects. Twenty two subjects carried two pathogenic mutations and 20 subjects carried one mutant allele, including one subject with one autosomal dominant mutation. The 235delC was the most common mutation accounting for 65.6% (42/64) GJB2 mutant alleles. When compared to other Asian populations, our subject cohort had higher frequency of 235delC mutation than the Japanese population. The GJB2 mutant alleles account for 23.7% (64/270) of all chromosomes responsible for nonsyndromic hearing impairment. Testing of the 4 most prevalent deleterious frame shift mutations(235delC, 299_300delAT, 176_191del16, and 560_605ins46) in this cohort detected 90% of all GJB2 mutant alleles. These results demonstrate that effective genetic testing of the GJB2 gene for patients and families with nonsyndromic hearing impairment is possible in the Chinese population. Since the most common 309kb GJB6 deletion is not detected and only one 1555 A>G mutation in mitochondrial DNA is detected in our patients, investigation of mutations in other nuclear genes and/or environmental factors responsible for nonsyndromic hearing impairment in the Chinese population is necessary.

Key words GJB2; mutation; non-syndromic; hearing impairment

Introduction

Hearing impairment is the most common neurosensory disorder in humans that has an incidence of approximately 1 in 1000 children. About two thirds of cases have a genetic etiology[1]. Hereditary deafness is genetically heterogeneous. Nonsyndromic deafness accounts for 60-70% of inherited hearing impairment and involves more than 100 different genes with autosomal dominant(DFNA), autosomal recessive (DNFB), X-linked(DFN), and maternal inheritance[2]. The most common cause for nonsyndromic autosomal recessive hearing loss is mutations in Connexin 26, a gap junction protein encoded by the GJB2 gene[3-10]. However, about 30% to 40% of hereditary deafness is syndromic, presenting with other clinical features in addition to hearing impairment.

Connexins are transmembrane proteins. Six

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monomers of connexin proteins associate to form a transmembrane hexameric gap junction hemi-channel called a connexon. Connexons embedded in the surfaces of adjacent cells associate to form an intercellular channel [11]. In the inner ear, connexin 26 can be in association with other connexins to form heteromeric connexons, and gap junction channels can be homotypic or heterotypic. The hair cells use connexin 26 gap junction channels to recycle the potassium ion as part of the mechanism of auditory signal transduction [12].

In deed, mutations in three connexin(Cx) genes, *GJB2* for Cx26, *GJB6* for Cx30, and *GJB3* for Cx31, have been identified and known to cause hearing impairment [13 – 15]. However, sequence analysis of the GJB2 gene in subjects with autosomal recessive hearing impairment has revealed a puzzling problem: a high number of patients carry only one mutant allele. Some of these families show clear evidence of linkage to the *DFNB1* locus, which contains two genes, *GJB2* and *GJB6*[3, 16]. Further analysis demonstrates that a deletion truncating the *GJB6* gene, encoding connexin 30, near GJB2 accompanies in trans in heterozygous affected subjects[17,18].

To date, more than 100 mutations, polymorphisms, and unclassified variants have been described in the *GJB2* to account for 10%-50% of molecular etiology in patients with nonsyndromic hearing impairment (http://davinci.crg.es/deafness). The mutation spectrum and prevalence of mutations vary significantly among different ethnic groups. Three mutations, 35delG, 167delT, and 235delC, are found to be the most frequent mutations in Caucasian, Ashkenazi Jewish, and Asian populations, respectively [3, 5-10, 13, 16, 19, 20]. A recent multicenter study reported that the 35delG mutation accounted for 72.44% of *GJB2* mutant alleles in 1,718 patients with biallelic DFNB1 mutations, including delGJB6-D13S1830 [21]. This group of patients consisted of 90% white. In Ashkenazi Jews, 35delG and 167delT account for 96% of the *GJB2* mutant alleles[22]. However, the 35delG mutation is rarely found in Asian patients. Instead, the 235delC mutation is the most prevalent in the Japanese, Korean, and Taiwanese [6, 10, 19, 23, 24]. Due to the broad mutation spectrum and prevalence of mutations in various ethnic groups, identification of mutations and their frequencies in a specific ethnic group is important in providing accurate genetic counseling and risk assessment for patients and families.

Although the majority of cases with hereditary hearing loss are caused by nuclear gene defects, in recent years, it has become clear that mitochondrial genes that are essential for energy metabolism can also cause hearing impairment. In fact, sensorineural hearing loss is one of the most prevalent and recognized clinical features of mitochondrial cytopathies [2, 25]. Mutations in mitochondrial DNA(mtDNA) can also cause non-syndromic hearing loss[25 – 27]. The most well studied mutation is the(1555 A>G) mutation in the mitochondrial 12S rRNA gene causing the nonsydromic hypersensitivity to ototoxic effects of aminoglycosides by increased binding of aminoglycosides to mitochondrial ribosomes, leading to the disruption of mitochondrial protein synthesis [28, 29]. Another recently identified mutation in the mitochondrial 12S rRNA gene is the 1494 T>C in the conserved stem structure of 12S rRNA [30]. Other nucleotide changes at position 961 in the 12S rRNA gene have been found to be associated with hearing loss, but their pathogenic mechanism in the predisposition of carriers to aminoglycoside toxicity is much less clear [31, 32]. Several mutations(7445 A>G, 7472insC, 7510 T>C, 7511 T>C, and 7512 T>C) in the mitochondrial $tRNA^{ser(UCN)}$ gene are also known to cause maternally inherited non-syndromic hearing loss (MIHL) by disturbing the tRNA structure and function [2, 25, 33].

The mtDNA 1555 A>G mutation accounts for a small fraction of patients with nonsyndromic hearing loss, that is usually post-lingual. The frequency has been reported to be between 0.6% to 2.5% among different Caucasian groups [34 – 38], and higher in Asian countries: 2.9%, 3%, and 5.3% in Chinese, Japanese, and Indonesian cohorts, respectively [39 – 41]. However, the incidence of 1555 A>G mutation in aminoglycoside induced ototoxicity is much higher. In a Japanese study, the 1555 A>G mutation was found in as high as 33% of patients with a history of aminoglycoside exposure [39]. In contrast, 13% of Chinese pediatric patients with aminoglycoside ototoxicity carry the 1555 A>G mutation [41].

In China, it is estimated that 30,000 babies are born with congenital hearing impairment in 20,000,000 live births every year [42]. However, the molecular etiology of Chinese deaf children has not been thoroughly investigated and effective genetic testing is yet to be offered. Since China is a big country with more than 56 different ethnic groups(including Han,

Zhuang, Man, Mongolian, Hui, Miao,Yi, Wa, Bai, Zang, Wei, Qiang, NaXi, etc) clustered in different parts of China, comprehensive genetic analysis of deaf children in different regions of China needs to be performed to obtain valid epidemiological information in order to provide effective genetic testing and accurate counseling. Recently, we screened for the mtDNA 1555 A>G mutation in Chinese deaf children in various regions of China and found that this mutation accounted for about 2.83% of nonsyndromic hearing impaired children [42]. Surprisingly, this mutation occurred at a much lower frequency in patients from Inner Mongolia when compared to other parts of China. To understand more about the genetic etiology of deafness among the Inner Mongolian population, we performed a comprehensive genetic analysis of the *GJB2* in a cohort of subjects attending the Chifeng Municipal Special Education School. We found that 38 of the 135(28.1%) patients with nonsyndromic hearing impairment carried confirmed pathogenic mutations and 4 harbored unclassified variants in the *GJB2*. Deletion of the GJB6 gene was not detected in the heterozygous *GJB2* mutation carriers.

Material and Methods

Patients and DNA samples

A total of 141 deaf students from 135 unrelated families attended the Chifeng Municipal Special Education School in Inner Mongolia, China, of which 135 probands from different families were included in this study. The Chifeng Municipal Special Education School is the only school for the deaf in the area. All children with moderate to profound bilateral hearing loss from the city of Chifeng and from the area within 500 km radius come to this school. More than 95% of the students participated in the study. The subject cohort consisted of 85 males and 50 females aging from 3 to 20 years (average 13.2±3.6 years). There were 94 Han, 31 Mongolian, 7 Man, and 3 Hui Chinese children in this group. The study was conducted according to a protocol approved by the Ethics Committee of the Chinese PLA General Hospital. Informed consents were obtained from legal guardians prior to collecting blood samples. Parents were interviewed for the age of hearing loss diagnosis, family history, maternal health during pregnancy, and the child's medical history including infection, head or brain injury, and use of aminoglycoside antibiotics. All subjects showed moderate to profound bilateral sensorineural hearing loss on audiograms. DNA specimens from all subjects and 100 race-matched students with normal hearing (determined by teachers and otolaryngologists) were sequenced for the presence of mutations and polymorphisms in the *GJB2* gene and in 12S rRNA and $tRNA^{Ser(UCN)}$ mtDNA genes. DNA was extracted from peripheral blood leukocytes using a commercially available DNA extraction kit(Watson Biotechnologies Inc, Shanghai, China).

Mutational analysis

DNA sequence analysis of the GJB2 gene was performed via PCR amplification of the coding exon plus approximately 50 bp of the flanking intron regions followed by Big Dye sequencing. The sequence results were analyzed using an ABI 3100 DNA sequencing machine (ABI, Foster City, USA.) running on ABI 3100 Analysis Software v.3.7 NT according to manufacturer's manual. Patients with one *GJB2* mutant allele were analyzed for the presence of *GJB6* gene deletion using the PCR method [17, 18]. A positive control provided by Balin Wu(Department of Laboratory Medicine, Children's Hospital and Harvard Medical School, USA.) was used for detection of *GJB6* gene deletion. Finally, part of the mitochondrial 12S rRNA gene containing the 1555 A>G mutation (nt611 to nt2007) and the tRNAser region(nt7148 to nt8095) were sequenced in all subjects.

Results

Among the 135 cases studied, 73 were pre-lingual hearing loss including 56 cases of congenital deafness. Twenty-eight cases were post-lingual hearing loss with an average age of onset at 2.93±1.48 years. The onset age of the remaining 34 cases were unclear. In addition, 38 cases (18 pre-lingual and 20 post-lingual) had clear history of aminoglycoside usage with an average age of hearing loss onset at 1.67 ±1.6 years. The average age of hearing loss onset in subjects without history of aminoglycoside usage was 0.681 ± 1.078 years, significantly lower than that in those with aminoglycoside usage($P<0.001$).

GJB2 mutations

Sequence analysis of the *GJB2* revealed that 22 subjects carried 2 confirmed pathogenic mutations and one had a R75W mutation, which has been reported to cause autosomal dominant syndromic deafness with palmoplanter keratoderma [43] (Table 1). Sixteen subjects, including the one with autosomal dominant

Table 1. Genotype of patients with mutations in GJB2 gene.

Allele 1			Allele 2			
Nucleotide Change	consequence or amino acid change	category	nucleotide change	consequence or amino acid change	category	Number of patientsd
c.235delC	frame shift	pathogenic	c.235delC	frame shift	pathogenic	14
c.235delC	frame shift	pathogenic	c.299_300delAT	frame shift	pathogenic	3
c.235delC	frame shift	pathogenic	c.176_191del16	frame shift	pathogenic	1
c.235delC	frame shift	pathogenic	c.257C>G	p.T86R [e](TM2)	pathogenic	1
c.560_605ins46	frame shift	pathogenic	c.560_.605ins46	frame shift	pathogenic	1
c.299_300delAT	frame shift	pathogenic	c.176_191del16	frame shift	pathogenic	2
(c.223C>T)	([a]R75W) [e]EC1 Autosomal dominant	aPathogenicPPK	(c.79G>A, c.341A>G)	(V27I,E114G)	polymorphism	1
c.235delC	frame shift	pathogenic	-			9
c.299_300delAT	frame shift	pathogenic	-			5
c.155_158delTCTG	frame shift	pathogenic	-			1
(c.592G>A)	([b]V198M) [e]TM4	novel	(c.79G>A, c.341A>G)	(V27I, E114G)	polymorphism	2
c.187G>T	[b]V63L [e](EC1)	reported	-			1
(c.458T>C)	([b]V153A) [e]EC2	novel	(c.608T>C)	(I203T)	polymorphism	1
c.109G>A	[c]V37I , [e] TM1	[c]see note	-			2
(c.109G>A)	([c]V37I)	[c]see note	(c.79G>A, c.341A>G)	(V27I,E114G)	polymorphism	1
c.79G>A, c.341A>G	V27I, E114G [e] (IC2)	polymorphism	-			44
c.79G>A, c.341A>G	V27I, E114G	polymorphism	c.79G>A, c.341A>G	V27I, E114G	polymorphism	2
c.341A>G	E114G	polymorphism	-			1
c.79G>A	V27I TM1	polymorphism	-			9

Novel alterations are in bold and italic. Mutations or polymorphisms with unknown phase are in parenthesis.
aR75W has been reported in autosomal dominant syndromic deafness with palmoplantar keratoderma (PPK) (Richard et al 1998b).
bV198M, V63L, and V153A are unclassified variantsl with unknown pathogenicity. V63L has been reported by (Hwa et al. 2003). V63A have been reported by Tang et al (2006 in press) as a novel alteration and V153I (not V153A) can be found in http://davinci.crg.es/deafness as a.polymorphism.
cV37I has been reported as a pathogenic mutation and as a polymorphism. In this report, we count it as a polymorphism (Table 3).
dAll patients listed here are from unrelated families.
eTM=transmembrane domain, EC=extracellular domain, IC=intracellular domain

R75W mutation, were heterozygous for one pathogenic mutant allele and 4 were heterozygous for one unclassified novel variant whose pathogenicity has not been determined(Table 1). In addition, 3 subjects carried the heterozygous allele of V37I, whose role as either a pathogenic mutation or a polymorphism has been in great debate [8, 19, 20, 24, 44-46]. Thus, 23 of the 135 unrelated deaf families (17.04%) in Chifeng City, Inner Mongolia, had confirmed molecular etiology of nonsyndromic hearing impairment(22 autosomal recessive and 1 autosomal dominant) in the GJB2.

Seven types of pathogenic mutations were identified, including 5 frameshift mutations (235delC, 299_300delAT, 176_191del16, 560_605ins46, and 155_158delTCTG) and 2 missense mutations (T86R and R75W) (Table 1). The most prevalent mutation in this subject cohort was 235delC, which has also been reported to be the most prevalent in other Asian populations [6, 19]. Fourteen subjects were homozygous for 235delC mutation: 5 compound heterozygous with another pathogenic mutation and 9 heterozygous carriers of 235delC mutation only(Tables 1). Four novel alterations were identified, including a frame shift pathogenic 155_158delTCTG mutation and three unclassified missense variants(V198M, V63L and V153A, see Tables 1 and 2). Overall, 64 mutant alleles (including the unclassified missense variants and one autosomal dominant, R75W, but excluding the V37I variant) were identified in 42 unrelated subjects. The 235delC alone accounted for 65.6% (42/64) of the total mutant alleles. Two mutations, 235delC and 299delAT accounted for 81.3% (52/64) of the *GJB2* mutations in

our patients, in comparison to 91% in a different Chinese population [46] and 97% in the Taiwanese population [23]. G45E and G4D were not detected in this group of subjects. Three T123N alleles were detected in our control but not in the test group.

The mutation spectra of the *GJB2* did not seem to vary among different ethnic groups in Chifeng area, with 235delC being the most common mutation in all groups. The 299_300delAT mutation was found in 7 Han, 2 Mongolian and 1 Hui subjects. The 560_605ins46 deleterious mutation was found in 1 Man subject. The 176_191del16 was detected in 2 Han and 1 Mongolian subjects, and the 155_158delTCTG in 1 Man subject. Four out of the 7(57%) Man subjects, and about 30% of subjects from other ethnic backgrounds carried the *GJB2* mutations. The difference of GJB2 detection rate among these 4 ethnic groups was not significant (χ=2.4893, P value 0.4772).

GJB2 polymorphisms

GJB2 gene analysis in the control subjects showed 4 deleterious mutations(Table 5), giving a carrier rate of 4%(4/100). Three T123N alleles and 8 V37I alleles were also detected.

Correlation of genotype with onset age of hearing loss and clinical phenotype

Excluding heterozygous patients with 1 mutant allele but including those with R75W, the average onset age of hearing loss in subjects with *GJB2* mutations was 0.97 ± 0.22 years, while that in those without *GJB2* mutations was 1.01 ± 1.37 years (P = 0.9689 and t= 0.039).

Deletion in GJB6 and 1555 A>G mutation

According to Del Castillo et al., a good proportion of patients heterozygous for one mutant allele in the *GJB2* has a 342 kb(now considered as 309 kb) deletion in the *GJB6* [17, 18, 47]. None of our heterozygous subjects with one *GJB2* mutant allele showed this deletion in the *GJB6* gene. The 1555 A>G mutation in the mtDNA 12S rRNA gene was detected in one subject who had a clear history of aminoglycoside usage. None of the remaining 37 subjects with history of aminoglycoside usage had mutations in the 12S rRNA gene. Mutations in $tRNA^{ser(UCN)}$ were not detected in any of our subjects.

Discussion

Previous reports have suggested that the prevalence of *GJB2* mutations among different ethnic groups varies. The carrier rate of deleterious *GJB2* mutation among the normal subjects in this study was 4%. The same carrier rate is 2% in Korea, 2.08% in Japan, 2.55% in Taiwan, 4.76% among Ashkenazi Jews, and 3.01%, in Midwestern United States. In our subjects, the most common mutation in Caucasians , i. e., 35delG, was not found. Instead, the 235delC mutation accounted for 65.6% of *GJB2* mutant alleles, supporting the notion that the 235delC mutation in the connexin 26 gene is the most prevalent mutation in Asian populations, including the Han Chinese. Four additional frameshift, pathogenic mutations were found, including one novel mutation c.155_158delTCTG. The 299delAT, 176_191del16, and 560_605ins46 mutations have all been reported in Taiwanese, Japanese and Korean patients [6, 10, 19, 23, 24]. These five frame shift mutations accounted for 90.6% (58/64) of all *GJB2* mutations found in this subject group. This is the highest level of mutation detection rate among Asian populations. Detection rates at approximately 41% and 57% have been documented in two Japanese reports, 67% in one Taiwanese paper and 73% in one Korean study(Table 2) [6, 10, 19, 23, 24]. In addition to the most common 235delC mutation, G45E accounts for 16% of the Japanese *GJB2* mutations, while G4D accounts for 10.6% of Taiwanese *GJB2* mutant alleles(Hwa et al. 2003; Ohtsuka et al. 2003). Neither of these mutations was detected in our subjects. The differences in *GJB2* mutation spectrum and prevalence among different Asian populations suggest that *GJB2* mutations may also be different among different ethnic groups in China. The top 6 largest ethnic groups of the Chinese nation are: Han, Man, Mongolian, Hui, Zhuang and Miao. The Han comprises the majority of the Chinese population(91.6%) and 70% of our study subjects. Rare deleterious mutations were detected in 4 ethnic groups in this study. There are no significant differences in *GJB2* mutation spectra among the different ethnic groups in this Chinese cohort, although the relative sample sizes of the non-Han subjects are small.

The missense mutation, T86R, was found in compound heterozygous with 235delC mutation in 1 subject. Although not listed in the *GJB2* mutation database(http://davinci.crg.es/deafness), this mutation has been reported in 3 Japanese patients [10]. The 15 year old Chinese female with R75W mutation in the current study reported no history of ototoxic medication exposure. She developed thickening and peeling of the skin at medial and lateral sides of both hands and feet

when she was 1 year of age. She was treated with traditional Chinese herbal medicines with no significant improvement. Audiometric testing showed moderate high frequency hearing loss in her father and normal hearing in her mother. Both parents had no skin lesions. *GJB2* sequencing of the parents showed they were not R75W mutation carriers, suggesting that the R75W in the subject was a de novel mutation. This mutation has been previously reported in association with autosomal dominant deafness and palmoplantar keratoderma[43]. The R75 is located at an evolutionarily conserved region of extracellular domain 1, where change of the highly positively charged arginine residue to highly hydrophobic aromatic amino acid tryptophan apparently distorts the structure and function of the connexin 26 protein molecule. Three missense variants; V63L, V153A, and V198M, are located in extracelluar domain 1, 2, and transmembrane span 4, respectively, of the connexin 26 protein. Although GJB2 mutation spectra from different Asian populations share the same most common 235delC mutation, few of the rare variants overlap(Table 2), suggesting that the rare variants have evolved recently in different geographic populations. The V153A and V198M are novel variants and not reported in the database (http://davinci.crg.es/deafness). However, V63L has been found in 1 Taiwanese patient [24]. These three variants likely contribute to the pathogenesis of deafness, because (a) they have been detected only in the patient group and not in 394 Japanese, 864 Taiwanese, and 494 Korean subjects, and(b) they are evolutionarily conserved from xenopus, mouse, rat, sheep, Orangutan and human. In fact, they are invariably valine at all three amino acid positions throughout evolution, except that V153 is a leucine in Xenopus (data not shown). These mutations were found heterozygous in 4 unrelated subjects who carried only one mutant allele. It is not clear if they represent autosomal dominant mutations, or they are autosomal recessive with the second mutant allele not yet identified. The second mutant allele may be either in the same gene (deep in introns, at the promoter site, or untranslated regions) or different genes(bigenic synergistic heterozygous mutations) [17, 48]. Heterozygous mutation carriers may be more susceptible to environmental factors that cause deafness [49]. Alternatively, these patients may be simply coincidental carriers whose deafness is caused by non-genetic environmental factors.

The pathogenicity of V37I is controversial. The V37I variant has been considered as a pathogenic mutation in the Japanese studies, but not found in any of the Korean control or patient populations [6, 10, 19]. In a recent multicenter study, the V37I mutation was found to be associated with mild to moderate hearing impairment (median 25-40 dB HL) [21]. This mutation was the second most common mutation in the Japanese, accounting for 21% of all GJB2 mutant alleles. It is found at lower frequencies in patients than in controls in Chinese and Taiwanese studies [23, 24, 46] (Table 3). In our study, V37I allele is present at 4% (8/200) in the control population, but only 1.1% (3/270) in the test group. The V37I variant does not appear to be a disease causing mutation among the Chinese. The discrepancies may be due to patients' genetic and environmental backgrounds. All our subjects with V37I are heterozygous without a second mutant allele. Our data do not support the pathogenicity of V37I variant, although the possible predisposition effect by V37I on patients who have been exposed to detrimental environmental factors cannot be ruled out. Table 3 also lists the frequencies of four common GJB2 polymorphisms, V27I, E114G, I203T, and T123N in Asian populations. Although allele frequencies among different studies are different, the difference between test and control groups is statistically significant for V27I, E114G, but not for I203T in this study ($P<0.001$ for V27I and E114G, and $P = 0.436$ for I203T). The T123N is an unclassified variant. It is counted as polymorphism in Taiwanese studies, but as a pathogenic mutation in Japanese and Korean studies. In one Taiwanese study, 1 T123N allele was found in 648 patient chromosomes and 15 T123N alleles in 864 control chromosomes. We found 3 T123N alleles in the control subjects but none in the test subjects. Like the Taiwanese study, these data do not support the pathogenicity of T123N. V27I, E114G, and I203T are considered as polymorphisms in all reported studies. The nonsense mutation Y136X has been detected at 10% and 14.3% among patients in two Japanese studies but not in other Asian populations.

Mutations in the GJB2 gene account for 10%-50% of autosomal recessive nonsyndromic hearing impairment world wide [50, 51]. Table 4 lists frequencies of GJB2 mutations reported in some patient populations. The ethnic background and patient ascertainment(multiple or single affected family members) account for the heterogeneity of mutations

Table 2. GJB2 mutation spectrum among Asian populations.

	Chinese 1 Our study	Control our study	Chinese2	Taiwanese1	Taiwanese2	Japanese1	Japanese2	Korean
[a]Total number of chromosomes	270	200	40	648	238	2454	70	294
Total mutant alleles identified	64 (100)		11 (100)	66 (100)	29 (100)	284 (100)	21 (100)	23 (100)
% of GJB2 mutation in total	23.7		27.5	10.2	12.2	11.6	30	7.8
235delC	42 (65.6)	1	9 (82)	38(57.6)	22 (76)	96(34)	10 (47.6)	15 (65)
299_300delAT	10 (15.6)	2	1 (9)	5 (7.6)	6 (21)	8 (2.8)	1 (4.8)	1 (4.3)
176_191del16	3 (4.7)			1 (1.5)		12 (4.2)	1 (4.8)	1 (4.3)
560_605ins46	2 (3.1)		1 (9)			1 (0.35)		
155_158del4	1 (1.6)							
T86R	1 (1.6)					3 (1.1)		
R75W b(AD)	1 (1.6)							
V198M	2 (3.1)							
V153A	1 (1.6)							
V63L	1 (1.6)			1 (1.5)				
G4D				7 (10.6)				
W77X				4 (6)				
F191L				3 (4.5)		4 (1.4)		
R184Q b (AD)				2 (3)	1 (3)			
R75Q b (AD)				2 (3)				
G130A				1 (1.5)				
V43M				1 (1.5)				
I30V				1 (1.5)				
[c]V37I	3 (1.1)	8		58		61 (21)	1 (4.8)	
G45E						45 (16)	1 (4.8)	
Y136X						30 (10)	3 (14.3)	
[d]T123N		3		1		11 (3.9)		1 (4.3)
R143W						8 (2.8)	4 (19)	1 (4.3)
I71T						4 (1.4)		
A49V						1 (0.35)		
S85P								2 (8.7)
35delG								2 (8.7)
E47[*]		1						
unknown								
references	This study	This study	(Shi et al. 2004)[e]	(Hwa et al. 2003)	(Wang et al. 2002)	(Ohtsuka et al. 2003)	(Abe et al. 2000)[f]	(Park et al. 2000)

Numbers in the parentheses are the percentages of mutant alleles in total GJB2 mutant alleles identified.

a total number of chromosome studied=number of patients x 2

b AD: Autosomal dominant mutations

c V37I was only considered pathogenic mutation in Japanese studies. It is not included in the calculation in other studies.

d T123N is an unclassified variant. It was counted as a polymorphism in Taiwanese1 study, but counted as a pathogenic mutation in Japanese and Korean studies. In Taiwanese study, 1 T123N allele was found in 648 patient chromosomes and 15 T123N alleles were found in 864 control chromosomes.

e Shi's study included 20 new born patients with severe nonsyndromic hearing impairment.

f Abe's study included families containing one or two affected siblings.

g E114G was considered as a pathogenic mutation in Park's study. It is not included in this Table.

Table 3. Comparison of allele frequencies of V37I, V27I, E114G, and I203T between patients and controls

variant	Our patients	Our controls	Chinese Patient/control	Japanese Patient/ control	Taiwanese patient/control	Korean/control
Total alleles	270 (100)	200 (100)	40(100)/100 (100)	70 (100)/192 (100)	648(100)/864 (100)	294(100)/200 (100)
V37I	3 (1.1)	8 (4)	0/3 (3)	1 (1.5)/2 (0.7)	58 (9)/100 (11.6)	0/0
V27I	62 (23)	79 (39.5)	6 (15)/39 (39)	0/75 (25.5)	149 (23)/319 (37)	97 (33)/80 (40)
E114G	51 (18.8)	57 (28.5)	3 (7.5)/31 (31)	0/25 (8.5)	121 (19)/275 (32)	90 (31)/40 (20)
I203T	2 (0.74)	6 (3)	0/0	0/16 (5.4)	42 (6.5)/39 (4.5)	0/3 (1.5)
T123N	0	3 (1.5)	0/1 (1)	0/0	1(0.15)/14 (1.62)	1 (0.34)/0
references	This study	This study	(Shi et al. 2004)	(Abe et al. 2000)	(Hwa et al. 2003)	(Park et al. 2000)

The numbers are number of alleles. The numbers in the parentheses are the percentage of the variant alleles in the total number of alleles.

Table 4. Comparison of frequencies of nonsyndromic deafness caused by mutations in GJB2 gene in different studies

	Inner Mongolia	Taiwanese 1	Taiwanese 2	Chinese	Korean	Japanese	US (BCM)	Australian	UK familial	French	German	European
Total patients	[a]135	169	324	20	147	35	610	74	142	104	147	422
Patients with GJB2 mutation	42 ([b]100, 31.1)	17 (100, 10)	48 (100, 15)	6 (100, 30)	16 (100, 11)	11 (100, 31)	113 (100, 18.5)	18 (100, 24)	61 (100, 43)	43 (100, 41)	30 (100, 20)	173 (100, 41)
2 mutations	22(52.4, 16.3)	12 (71, 7)	19 (40, 5.9)	5 (83, 25)	7 (44, 4.8)	9 (82, 25.7)	63 (56, 10.3)	10 (56, 13.5)	45 (74,32)	35 (81, 33.7)	21 (70, 14.3)	129 (75, 30.6)
1 mutation[c]	20 (46.6, 14.8)	5 (29, 3)	29 (60, 9)	1 (17, 5)	9 (56, 6.1)	2 (18, 5.7)	50 (44, 8.2)	8 (44, 10.8)	16 (26, 11)	8 (19, 7.7)	9 (30, 6.1)	44 (25, 10.2)
Total mutant alleles	64 ([d]23.7)	29 (8.6)	66 (10)	11 (27.5)	23 (7.8)	20 (29)	176 (14.4)	28 (18.9)	106 (37)	78 (37.5)	51 (17.1)	302 (35.8)
references	This study	(Wang et al. 2002)	(Hwa et al. 2003)	(Shi et al. 2004)	(Park et al. 2000)	(Abe et al. 2000)	(Tang et al. 2006)	(Wilcox et al.2000)	(Hutchin and Cortopassi 2000)	(Denoyelle et al. 1999)	\(Gabriel et al. 2001)	(del Castillo et al.

[a] numbers are number of patients, including the allele of autosomal dominant mutations

[b] The numbers in the parenthesis stand for the percentage of patients in total number of deaf patients and patients carrying GJB2 mutations (with two or 1 mutation), respectively, for the first and the second number.

cinclude the patients carrying autosomal dominant mutations and unclassified variants

dPercentage of mutant alleles identified in total number of chromosomes, for example, 64/274=23.7

(Table 2) and the proportion of patients with two GJB2 mutant alleles. Patient populations containing more unrelated subjects without family history have lower chance of detection of two GJB2 mutant alleles and higher percent one mutant allele detection than related subjects. For example, double GJB2 mutant alleles are found in approximately 5% in Korean, 10% in US and 14% in German patient populations, respectively [6, 9, 52], whereas as high as 34% patients carry double *GJB2* mutant alleles in studies where patients were ascertained through strong family history [25, 53]. In our study, 22 of 135 unrelated patients(16.3%) had two confirmed pathogenic mutations, higher than reported by the two previous Taiwanese studies(7% and 6%), but lower than the Japanese reports on families.

A significant portion of patients with *GJB2* mutations have only one mutant allele. This also varies among different populations and, in general, is in reverse correlation with the proportion of patients harboring two mutant alleles(Table 4). Excluding the known autosomal dominant mutation R75W, but

Table 5. GJB2 gene mutation in 100 normal hearing individuals from Northern China

Allele 1			Allele 2			
Nucleotide Change	consequence or amino acid change	category	nucleotide change	consequence or amino acid change	category	Number of patients[d]
c.235delC	frame shif	pathogenic				1
c.299_300delAT	frame shift	pathogenic				1
c.299_300delAT	frame shift	pathogenic	c.79G>A, c.341A>G	V27I, E114G	polymorphism	1
c.608T>C	I203T	polymorphism				3
c.608T>C	I203T	polymorphism	c.79G>A, c.341A>G	V27I, E114G	polymorphism	3
c.109G>A	V37I TM1					5
c.109G>A	V37I		c.79G>A, c.341A>G	V27I, E114G	polymorphism	3
c.171G>T	[a]Q57H EC1	novel				1
c.139G>T	E47* EC1	novel	c.79G>A, c.341A>G	V27I, E114G	polymorphism	1
c.438C>T	[c]F146F TM3	polymorphism	c.79G>A, c.341A>G	V27I, E114G	polymorphism	1
c.341A>G	E114G IC2	polymorphism				1
c.79G>A	V27I TM1	polymorphism				6
c.79G>A	V27I	polymorphism	c.79G>A	V27I	polymorphism	2
c.79G>A, c.341A>G	V27I,E114G	polymorphism	c.79G>A, c.341A>G	V27I, E114G	polymorphism	7
c.79G>A, c.341A>G	V27I,E114G	polymorphism				24
c.79G>A, c.341A>G	V27I,E114G	polymorphism	c.79G>A	V27I	polymorphism	9
c.79G>A, c.368C>A	V27I, [b]T123N IC2	polymorphism				2
c.79G>A, c.368C>A	V27I,T123N	polymorphism	c.79A>G	V27I		1

[a]Both Q57H is novel with unknown pathogenicity and E47* nonsense mutation.

[b] T123N is described as unknown mutation in http://www.crg.es/deafness.

[c] F146F is a polymorphism which is not described. dTM=transmembrane domain, EC=extracellular domain, IC=intracellular domain

including the 4 subjects with unclassified variants, we detected 19(14.07%) cases with one *GJB2* mutant allele. Carriers of a single mutation in *GJB2* demonstrate evidence of reduced hair-cell function [54]. Thus, it is possible that these carriers are more likely than non-carriers to develop hearing impairment in the presence of other genetic defects or environmental factors [49]. Indeed, a common 342 kb deletion (now considered a 309 kb deletion) involving the coding region of connexin 30(*GJB6* gene) protein upstream of the GJB2 gene has been identified and found to account for up to 10% of DFNB1 alleles [18, 47]. In addition, *GJB2* mutations may act synergistically in the presence of mtDNA 1555 A>G mutation with aminoglycoside induced ototoxicity [55]. In this study, deletion in the *GJB6* was not detected in any of our subjects. The 1555 A>G mutation was found in a subject who had a history of aminoglycoside usage. Hwa et al., in studying Taiwanese prelingual deaf patients carrying one *GJB2* mutant allele, did not find any deletions or mutations in the *GJB6*, nor did they find 1555 A>G mutation in mtDNA, consistent with our results [24].

In summary, this study revealed a unique *GJB2* mutation spectrum in Chinese patients with nonsyndromic hearing impairment. The 235delC mutation is the most frequent mutations in deaf patients in Inner Mongolia, China. Testing for 235delC and 299_300delAT mutations detected 81.3% of *GJB2* mutant alleles. Considering the small sample size, it is possible that specific sub-ethnic group prevalent mutations within the Chinese population exist but are not demonstrated by this study. In order to provide effective genetic testing and accurate counseling, it is necessary to study the genetic etiology of hearing impairment among sub-populations in China. Furthermore, the molecular defects of more than 70% of the patients with nonsyndromic hearing impairment in China remain to be identified.

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